ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2011, p. 917–920 Vol. 55, No. 2
0066-4804/11/$12.00 doi:10.1128/AAC.00491-10

ISAba825, a Functional Insertion Sequence Modulating Genomic Plasticity and bla_{OXA-58} Expression in *Acinetobacter baumannii*[▽]

Pablo Ravasi, Adriana S. Limansky, Ramiro E. Rodriguez, Alejandro M. Viale, and María A. Mussi*

Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET) and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Rosario, Argentina

Received 10 April 2010/Returned for modification 15 July 2010/Accepted 12 November 2010

ISAba825, an insertion sequence found inactivating *Acinetobacter baumannii carO*, was tagged with a kanamycin (Kn) resistance cassette. ISAba825::Kn effectively transposed in *A. baumannii*, showing preference for short, AT-enriched target sequences, generating 6- to 9-bp target duplications. Additionally, we detected the presence of ISAba825 upstream of a plasmid-borne bla_{OXA-58} gene, generating a hybrid promoter largely enhancing its expression and leading to carbapenem resistance. Overall, a role for ISAba825 in carbapenem resistance modulation in *A. baumannii* is proposed.

Acinetobacter baumannii is an important opportunistic pathogen responsible for a variety of nosocomial infections (6, 21, 22, 26). It can rapidly evolve multidrug resistance (MDR) when confronted with antibiotic therapy (4, 6, 21, 26), and in particular, the emerging resistance to carbapenems among nosocomial strains represents a major concern worldwide (4, 20, 22). One of the mechanisms proposed to play a significant role in carbapenem resistance in *A. baumannii* is the expression of OXA-type carbapenemases (6, 7, 15, 16, 21, 26). Other mechanisms, such as alterations in outer membrane (OM) permeability, can also contribute to carbapenem resistance in this organism (5, 18). In this context, we recently reported that the loss of the OM channel CarO as a result of the natural insertional inactivation of its coding gene by insertion sequence (IS) ISAba825 or ISAba125 correlated with reduced susceptibility to carbapenems (18). One of these ISs, ISAba825, recently assigned to the IS*982* family (http://www-is.biotoul.fr), is composed of an 876-bp open reading frame (ORF) coding for a DDE-type transposase bounded by a perfect 17-bp inverted repeat (IR) (18). ISAba825 generated a 7-bp duplication (ATCGTTA) at the insertion site within *carO* (18). It is well known that ISs can cause insertion mutations and genome rearrangements and enhance the spread of resistance and virulence determinants within pathogenic species (2, 9, 11, 13, 17, 19, 23). In this work, we evaluated the impact of ISAba825 in modulating *A. baumannii* genome plasticity and carbapenem resistance.

To follow ISAba825 transposition, we tagged the element with a kanamycin (Kn) resistance cassette (Fig. 1A) and subcloned it into the plasmid pKNOCK, a suicide vector in *A. baumannii* (1). To evaluate the ability of ISAba825::Kn to transpose in *A. baumannii*, we first transformed an *A. baumannii* ATCC 17978 strain containing the Amp^r plasmid pWH1266 (which can replicate in *A. baumannii* and *Escherichia coli* [8]) with pKNOCK ISAba825::Kn. The rationale was that, as pKNOCK is unable to replicate in *A. baumannii*, Kn^r bacteria would arise, among other possible events, from ISAba825::Kn transposition to the chromosome, to ATCC 17978 endogenous plasmids pAB1 and pAB2 (27), or to pWH1266 (step 1). Step 2 involved plasmid extraction from these Kn^r colonies, transformation of *E. coli* DH5α or ATCC 17978 competent cells, and selection in LB agar plates containing Kn or Kn and Amp to isolate plasmids containing ISAba825::Kn insertions. At this step, we observed Kn^r transformants only when ATCC 17978 was used as the recipient for transformation (albeit none of them were Amp^r), suggesting that in *A. baumannii* there was effective transposition into plasmids other than pWH1266, which appeared to be nonreplicative in *E. coli*.

Putative ISAba825::Kn transposition events were analyzed using plasmid extractions from 40 ATCC 17978 Kn^r colonies, and in each of them the precise insertion site was determined by DNA sequencing (Maine DNA Sequencing Facility) of the IS immediate external neighboring regions by using primers ISout1 and ISout2 (Table 1). Reads of up to 500 bp at either side of ISAba825::Kn revealed 5 different IS insertions, all of them in the indigenous plasmids pAB1 and pAB2 (Fig. 1B and C). AT-enriched short target site duplications bounding the IRs were observed in all these cases, consistent with ISAba825::Kn transposition events (Fig. 1C). It is noteworthy that the target duplications ranged from 6 to 9 bp (Fig. 1C). It is commonly assumed that the length of the duplication is characteristic for a given element. However, certain ISs can generate duplications of atypical length, presumably reflecting small variations in the geometry of the transposition complex (13). Therefore, and although transposition was not tested in a *recA*-deficient strain, all the evidence described above strongly indicates effective transposition of ISAba825::Kn in *A. baumannii* ATCC 17978.

We evaluated next whether ISAba825 could be found in close proximity to any member of the different OXA groups in carbapenem-resistant strains of our collection by using combinations of forward and reverse primers specific for ISAba825 (Table 1) and the different *oxa* genes, respectively (14). From a total of 10 strains screened, a PCR amplification band of approximately 1,500 bp was obtained only for strain Ab880 by

* Corresponding author. Mailing address: Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Rosario, Argentina. Phone: 54-341-4350661. Fax: 54-341-4390465. E-mail: mussi@ibr.gov.ar.

▽ Published ahead of print on 22 November 2010.

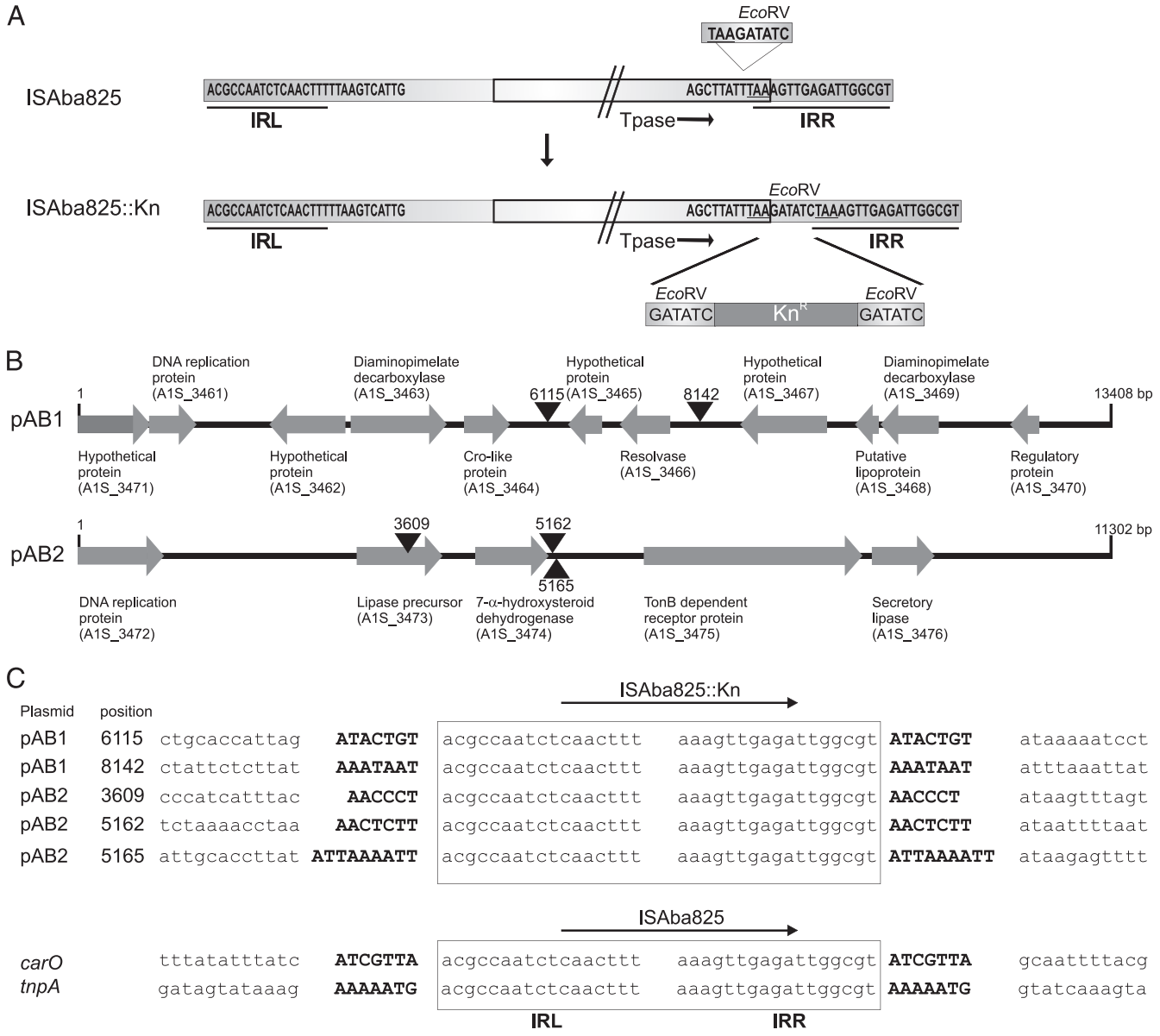

FIG. 1. (A) Construction of ISAba825::Kn. First, we generated an EcoRV site immediately downstream of the transposase (Tpase) gene and upstream of the right inverted repeat (IRR) of ISAba825 by PCR using primers 825TF and 825TR (Table 1). As the Tpase stop codon TAA is within the IRR, we introduced another TAA codon upstream of the EcoRV site to maintain both the original transposase sequence and the IRR structure. Afterwards, a kanamycin resistance cassette (Kn^R) bounded by two EcoRV sites was generated by PCR using primers Kn*Eco*RVF and Kn*Eco*RVR (Table 1), employing plasmid pBBR1MCS5 (10) as a template, and was inserted into the equivalent site present in ISAba825-EcoRV. The single TAA site in ISAba825 and the two TAA sites in ISAba825::Kn are underlined. The Tpase gene is boxed and the direction of transcription indicated by an arrow. The IRL and IRR are also underlined. The scheme is not drawn to scale. (B) Target sites of ISAba825::Kn insertions in linearized representations of ATCC 17978 endogenous plasmids pAB1 (GenBank accession number NC_009083) and pAB2 (GenBank accession number NC_009084). Genes and their corresponding directions of transcription are indicated by horizontal arrows. The locus tag of each gene is indicated in parentheses below the gene description. Dark arrowheads indicate ISAba825::Kn insertions at the corresponding nucleotide positions in the plasmids. Arrowheads above and below lines indicate IS insertions in opposite orientations. The scheme is not drawn to scale. (C) ISAba825::Kn and ISAba825 target sequences. Target site duplications (bold letters) and flanking regions of ISAba825::Kn insertions in pAB1 and pAB2 and ISAba825 insertions in *carO* and *tnpA* are shown. IR regions are boxed, and arrows indicate the Tpase transcription direction.

using primers 825TR and OXA-58R (Table 1). Sequence analysis revealed that an insertion of ISAba825 truncated the transposase gene (*tnpA*) of an ISAba3-like element located upstream of a bla_{OXA-58} gene (Fig. 2A). As in other cases, the bla_{OXA-58} gene was bounded by two ISAba3 elements (24, 25). Southern blot analysis showed that this arrangement was present in a plasmid (not shown), designated pAb880. Electrotransformation of pAb880 into ATCC 17978 resulted in 32- and 64-fold increments in meropenem (MER) and imipenem (IPM) MICs, respectively, compared to those of nontransformed bacteria (Table 2). This outcome suggested that the ISAba825 bla_{OXA-58} arrangement carried in pAb880 most

TABLE 1. Oligonucleotide primers used in PCR, sequencing, and 5′ RACE analysis

Primer	Sequence (5′→3′)[a]	Reference or source
ISout1	TCGGTTAAAGCAGGTGGA	This work
ISout2	CTTTATGCTTCCGGCTCG	This work
825TF	ACGCCAATCTCAACTTTTTAAGTCATTG	This work
825TR	ACGCCAATCTCAACTTTA*GATATC*TTAAATAAGCT	This work
OXA-58F	AAGTATTGGGGCTTGTGCTG	20
OXA-58R	TACGACGTGCCAATTCTTGA	20
IS	ACGCCAATCTCAACT	18
Kn*Eco*RVF	AAGTGC*GATATC*GGATGAATGTCAGCTAC	This work
Kn*Eco*RVR	TTGTTC*GATATC*GTGAGGGTTAATTGCG	This work
bla_{OXA-58}F_RT	TAGAGCGCAGAGGGGAGAAT	This work
bla_{OXA-58}R_RT	CATCACCAGCTTTCATTTGC	This work
*recA*F_RT	TACAGAAAGCTGGTGCATGG	This work
*recA*R_RT	TGCACCATTTGTGCCTGTAG	This work
5′RACE 1144	GACTCATACTATGCTCAGCAC	This work
5′RACE 1079	TTAATAATTTCATGATATACAAC	This work
5′RACE 1018	AAGCCGATTGGATTTTGATAA	This work
PISAba825F	*GGATCC*ATCCTGACCATAATGTG	This work
PISAba3F	*GGATCC*ATCACTGAGGCAGGTTG	This work
Pbla_{OXA-58}R	*GGATCC*TACACTCAAACTTCTAATTC	This work

[a] Restriction endonuclease sites are indicated in italics.

probably contributed to carbapenem resistance. 5′ rapid amplification of cDNA ends (5′ RACE) analysis identified the presence of a transcript initiating 119 bp upstream of the start codon of the bla_{OXA-58} gene, thus defining a hybrid promoter (ISAba825-ISAba3-like) in which the −10 region is within the ISAba3-like element and the −35 region is located within the ISAba825 left IR (IRL) (Fig. 2B). In this context, previous reports have also shown that insertion of different ISs within the 5′ ISAba3 element can result in alternate hybrid promoters modulating bla_{OXA-58} expression (3, 25). We then tested the contribution of the ISAba825-ISAba3-like hybrid promoter to bla_{OXA-58}-mediated carbapenem resistance by comparing the IPM and MER MICs of ATCC 17978 bacteria transformed with plasmid pWHP825 (pWH1266 bearing bla_{OXA-58} under the control of the ISAba825-ISAba3-like hybrid plus the previously described ISAba3-derived promoter [3]), pWHP3 (pWH1266 bearing bla_{OXA-58} lacking the hybrid promoter but preserving the ISAba3-derived promoter), or pWH1266 alone (Fig. 2B). As seen in Table 2, bacteria harboring pWHP825 exhibited MER and IPM MICs 64- and 128-fold higher, respectively, than those of cells harboring pWH1266. In contrast, transformation of bacteria with pWHP3 only doubled the MIC values for these carbapenems. In agreement, transcript levels of bla_{OXA-58} in pWH825-containing cells were 7-fold higher than those in pWHP3-containing cells and about 100-fold higher than those in bacteria bearing pWH1266 (Table 2). Thus, the ISAba825-ISAba3-like hybrid promoter-directed bla_{OXA-58} overexpression constitutes a likely mechanism leading to carbapenem resistance acquisition in *A. baumannii*.

Finally, the study of the distribution of ISAba825 by PCR

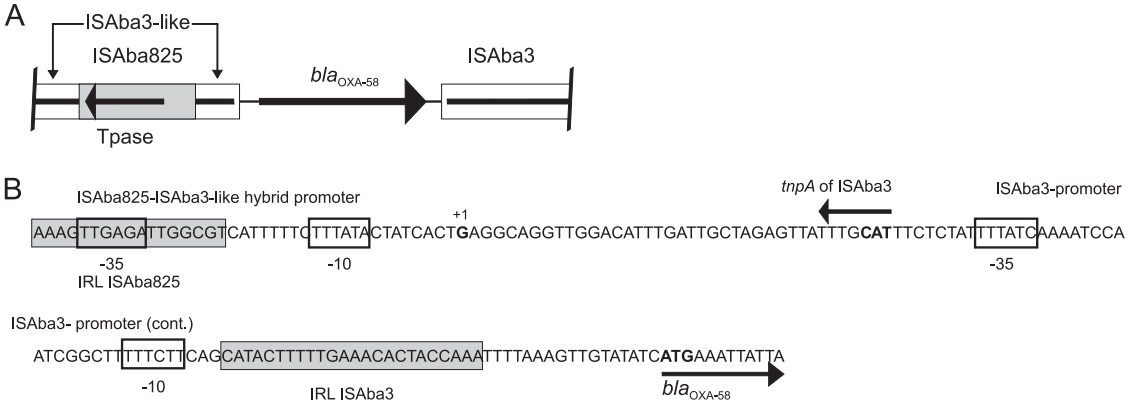

FIG. 2. ISAba825 insertion generated a hybrid promoter driving bla_{OXA-58} overexpression in the clinical *A. baumannii* strain Ab880. (A) Schematic representation of the genetic structure resulting from ISAba825 insertion within the ISAba3-like element located 5′ upstream of the bla_{OXA-58} gene present in plasmid pAb880. Genes and their corresponding transcription orientations are indicated by horizontal arrows. The boundaries of the sequenced fragment are indicated by vertical bars at the edges. The figure is not drawn to scale. (B) Promoter regions for bla_{OXA-58} in the arrangement described above. The −35 and −10 motifs inferred for each of the different promoters are boxed, and the transcription initiation site (G in bold) resulting from the hybrid promoter (as determined by 5′ RACE-PCR) is indicated by +1. Promoter prediction was done using BPROM (SoftBerry). The different ATG codons for bla_{OXA-58} and *tnpA* are indicated in bold, and the corresponding directions of transcription are shown by arrows. The inverted repeats of the different IS elements are shaded gray. cont., continued.

TABLE 2. ISAba825-mediated bla_{OXA-58} overexpression promotes carbapenem resistance in *A. baumannii*

A. baumannii strain	MIC (μg/ml)		bla_{OXA-58}/*recA* transcript ratio[c]
	IPM	MER	
Ab880	16	16	ND
ATCC 17978	0.25	0.25	ND
ATCC 17978/pAb880	16	8	ND
ATCC 17978/pWH1266	0.25	0.25	1.0
ATCC 17978/pWHP3[a]	0.5	0.5	16.4 ± 1.3
ATCC 17978/pWHP825[b]	32	16	118 ± 7

[a] pWHP3, plasmid directing expression of bla_{OXA-58} under an ISAba3 promoter (Fig. 2), constructed by cloning a PCR fragment amplified with primers PISAba3F and Pbla_{OXA-58}R (Table 1) in the BamHI site of pWH1266.

[b] pWHP825, plasmid directing expression of bla_{OXA-58} under ISAba825-ISAba3 hybrid and ISAba3-derived promoter sequences (Fig. 2), constructed by cloning a PCR fragment amplified with primers PISAba825F and Pbla_{OXA-58}R (Table 1) in the BamHI site of pWH1266. All constructions were verified by sequencing.

[c] Relative abundances of bla_{OXA-58} transcripts. For each RNA sample, bla_{OXA-58} transcripts levels were normalized to the corresponding *recA* levels, used as an internal control for constitutively expressed genes. Values obtained for ATCC 17978/pWH1266 were taken as 1. Total RNA was extracted from overnight cultures of the indicated bacteria grown in LB liquid media at 37°C. Primers bla_{OXA-58}F_RT/bla_{OXA-58}R_RT and *recA*F_RT/*recA*R_RT were used for reverse transcription-quantitative PCR (RT-qPCR) (12) estimations of bla_{OXA-58} and *recA* transcript levels, respectively. The data shown are the means ± standard errors of the means (SEM) of 4 replicates. ND, not determined.

using primer IS (Table 1) showed its presence only in carbapenem-resistant *A. baumannii* isolates of our collection and failed to detect it in other representatives of the genus *Acinetobacter*, including 2 different strains of *A. radioresistens*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. tandoii*, *A. bouvetii*, *A. tjernbergiae*, *A. grimontii*, *A. towneri*, and *A. gerneri*. Overall, the results described above, in addition to the inactivation of *carO*, lead us to propose a role for ISAba825 in the modulation of carbapenem resistance in *A. baumannii*.

Nucleotide sequence accession numbers. The ISAba825::Kn sequence (Fig. 1A) was deposited in GenBank under accession number HM068377, and the DNA sequence of the ISAba825-containing ISAba3-like element (shown in brackets in Fig. 2A) was deposited under accession number HM068378.

We are indebted to M. Vaneechoute and R. J. Seviour for providing strains representative of the different species within the *Acinetobacter* genus. We also thank L. Orsaria, N. Bologna, J. Palatnik, and G. Gago for their generous help in 5′ RACE and RT-qPCR experiments.

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, CONICET, and Departamento de Salud Pública, Municipalidad de Rosario, Rosario, Argentina). M.A.M., A.M.V., and R.E.R. are career investigators of CONICET. A.S.L. is a career investigator of the National University of Rosario.

REFERENCES

1. **Alexeyev, M. F.** 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. Biotechniques **26:**824–826, 828.
2. **Aubert, D., T. Naas, C. Heritier, L. Poirel, and P. Nordmann.** 2006. Functional characterization of IS1999, an IS4 family element involved in mobilization and expression of β-lactam resistance genes. J. Bacteriol. **188:**6506–6514.
3. **Chen, T., R. Wu, M. Shaio, C. Fung, and W. Cho.** 2008. Acquisition of a plasmid-borne bla_{OXA-58} gene with an upstream IS1008 insertion conferring a high level of carbapenem resistance to *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **52:**2573–2580.
4. **Coelho, J. M., et al.** 2006. Occurrence of carbapenem-resistant *Acinetobacter baumannii* clones at multiple hospitals in London and southeast England. J. Clin. Microbiol. **44:**3623–3627.
5. **del Mar Tomás, M., et al.** 2005. Cloning and functional analysis of the gene encoding the 33- to 36-kilodalton outer membrane protein associated with carbapenem resistance in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **49:**5172–5175.
6. **Gordon, N. C., and D. W. Wareham.** 2010. Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. Int. J. Antimicrob. Agents **35:**219–226.
7. **Héritier, C., A. Dubouix, L. Poirel, N. Marty, and P. Nordmann.** 2005. A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolysing oxacillinase OXA-58. J. Antimicrob. Chemother. **55:** 115–118.
8. **Hunger, M., R. Schmucker, V. Kishan, and W. Hillen.** 1990. Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. Gene **87:**45–51.
9. **Kato, N., K. Yamazoe, C. G. Han, and E. Ohtsubo.** 2003. New insertion sequence elements in the upstream region of *cfiA* in imipenem-resistant *Bacteroides fragilis* strains. Antimicrob. Agents Chemother. **47:**979–985.
10. **Kovach, M. E., et al.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
11. **Lin, H., T.-Y. Li, M.-H. Xie, and Y. Zhang.** 2007. Characterization of the variants, flanking genes, and promoter activity of the *Leifsonia xyli* subsp. *cynodontis* insertion sequence IS*1237.* J. Bacteriol. **189:**3217–3227.
12. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods **25:**402–408.
13. **Mahillon, J., and M. Chandler.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. **62:**725–774.
14. **Marchiaro, P., et al.** 2008. A convenient microbiological assay employing cell-free extracts for the rapid characterization of Gram-negative carbapenemase producers. J. Antimicrob. Chemother. **62:**336–344.
15. **Marqué, S., et al.** 2005. Regional occurrence of plasmid mediated carbapenem-hydrolyzing oxacillinase OXA-58 in *Acinetobacter* spp. in Europe. J. Clin. Microbiol. **43:**4885–4888.
16. **Merkier, A. K., et al.** 2008. Polyclonal spread of bla_{OXA-23} and bla_{OXA-58} in *Acinetobacter baumannii* from Argentina. J. Infect. Dev. Ctries. **2:**235–240.
17. **Mugnier, P., L. Poirel, and P. Nordmann.** 2009. Functional analysis of insertion sequence ISAba1, responsible for genomic plasticity of *Acinetobacter baumannii*. J. Bacteriol. **191:**2414–2418.
18. **Mussi, M. A., A. S. Limansky, and A. M. Viale.** 2005. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii*: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins. Antimicrob. Agents Chemother. **49:**1432–1440.
19. **Nagai, T., L. S. Phan Tran, Y. Inatsu, and Y. Itoh.** 2000. A new IS*4* family insertion sequence, IS*4Bsu1*, responsible for genetic instability of poly-γ-glutamic acid production in *Bacillus subtilis*. J. Bacteriol. **182:**2387–2392.
20. **Pasterán, F., et al.** 2006. Emergence of PER-2 and VEB-1a in *Acinetobacter baumannii* strains in the Americas. Antimicrob. Agents Chemother. **50:** 3222–3224.
21. **Perez, F., et al.** 2007. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **51:**3471–3484.
22. **Perez, F., A. Endimiani, and R. A. Bonomo.** 2008. Why are we afraid of *Acinetobacter baumannii*? Expert Rev. Anti Infect. Ther. **6:**269–271.
23. **Poirel, L., J. W. Decousser, and P. Nordmann.** 2003. Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a bla_{CTX-M}-lactamase gene. Antimicrob. Agents Chemother. **47:**2938–2945.
24. **Poirel, L., et al.** 2005. OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **49:**202–208.
25. **Poirel, L., and P. Nordmann.** 2006. Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-58} in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **50:** 1442–1448.
26. **Poirel, L., and P. Nordmann.** 2006. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin. Microbiol. Infect. **12:**826–836.
27. **Smith, M., et al.** 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes Dev. **21:**601–614.